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(54) Method for preparing transformed plant.

(57) A transformed plant of Gramineae is prepared by culturing an anther of Gramineae in a callus induction medium and, at a stage immediately before the microspore begins to divide or during the initial division, transducing a genetic substance into the microspore cell through a pore formed by a laser pulse thereby to express genetic information of the genetic substance.

According to the present invention, it is unnecessary to prepare protoplasts and therefore, time and operations for transformation can be greatly reduced. Since haploid cells are transformed, the character transduced is conveyed without separating at a later generation. In addition, difficulties in experiments between species and strains are minimized so that it is easy to apply the present invention to practical species. According to the present invention, large pores can be formed as compared to the electroporation method so that DNA or substances having a large molecular weight can be introduced.

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The present invention relates to a method for preparing a transformed plant of the family Gramineae and a method for transforming a plant of the family Gramineae.

As a method for transforming a plant, genetic transduction utilizing Agrobacterium has been established. However, Agrobacterium fails to infect grasses of the family Gramineae. Thus other techniques have been attempted.

As one of the techniques, direct transduction of vector DNA has been studied. For example, methods for transformation by means of electroporation, particle gun, polyethylene glycol or microinjection have been established. It is therewith possible to transduce a gene into a protoplast or callus in corns [Nature, 319, 791 (1986)], grasses [Mol. Gen. Genet., 204, 204 (1986)], wheat [Mol. Gen. Genet., 199, 178 (1985)] and pasture [Mol. Gen. Genet., 199, 178 (1985)]. In all or these methods, obtaining transgenic plant is greatly restricted by the difficulty of culturing protoplasts and the complicated handling. As an example where a gene is transduced into a plant, there is a report that a plasmid is injected into young seedling rye by microinjection and the gene is expressed in seeds derived from the plant [Nature, 325, 274 (1986)]. However, this technique has not yet been established as a general method for transducing a gene into a plant efficiently.

There is also reported a method for preparing a transformed plant using the transduction system in Gramineae by electroporation into protoplasts [published unexamined Japanese patent application No. 1-18179]. In many cereal plants,the regeneration from protoplasts has not been established. In addition, many selection cultures and long periods of time are required for the preparation of protoplasts. It is also the actual situation that protoplast culture is applicable only to a part of the species and cultivars having excellent tissue culture property, even though they are the same crop.

A method for transformation by laser perforation is currently utilized for transformation of animal cells and used in preliminary experiments on plant tissues and cells [Seber et al., Plant Cell Tissue and Organ Culture, 12, 219 (1988)], and experiments on organelles[West German patent application 3,707,111A]. However, it is unknown whether microspores of a plant can be transformed by the method described above. There is also reported a method for preparing a transgenic plant using the gene transduction system into sweet corn embryo employing laser technology [published unexamined Japanese patent application No. 2-9378]. According to this method, however, the thus obtained transformant is a chimera. So far no method has been established for obtaining non-chimeric, transformed cereal plants.

As described above, many attempts have been made on transformation of plants but transformation has not yet been successfully carried out for monocotyledons, especially Gramineae, although this has long been desired.

Thus, the technical problem underlying the present invention was to provide a method for the transformation of monocotyledonous plants, preferably Gramineae. The solution to this technical problem is provided by the embodiments characterized in the claims.

Thus, the present invention relates to a method for obtaining transgenic plants of the family Gramineae which comprises efficiently transducing a foreign gene into microspores of the plants belonging to the family Gramineae.

That is, the present invention provides a method for transforming Gramineae which comprises culturing an anther of Gramineae in a callus induction medium and, at a stage immediately before the enveloped microspore begins to divide or during the initial division, transducing a genetic substance to the pollen cell through a pore formed by a laser pulse. The present invention also provides a method for obtaining a transgenic plant which comprises expressing genetic information of the genetic substance in the transformant.

Hereafter the present invention is described in detail.

The method of the present invention can generally be performed by culturing an anther in a callus induction medium and, suspending suitable microspores at a stage immediately before the single cells begin to divide or during the initial division, in a solution, typically an aqueous solution, containing a genetic substance bearing genetic information to be transduced. Then, a laser device is focused on one of the microspore cells and the laser is excited to form pores in the cell envelope. Through the pores, the genetic substance is introduced into the cell. The cell is cultured in callus induction medium to form a callus and/or an embryoid. The callus and/or embryoid is then subcultured on medium for regeneration. The transgenic plant can thus be obtained.

In order to culture appropriate microspore cells at a stage immediately before the single cells begin to divide or during the initial division by culturing the anther of Gramineae in callus induction medium, the anther is cultured in a medium chosen based on the property of anther to be cultured, from modified MS medium [Carlsberg Res. Commun., 52, 393 (1987)], FHG medium [Kasha et al., XIX Stadler Genetics Symp., 213 (1989)], Clapham I, II, III medium [Z. Pflanzenzucht, 69, 142 (1973)], Foroughi-Weir et al.

medium [Z. Pflanzenzucht, 77, 198 (1976)] and modified media thereof. The temperature for the culture varies depending on the anther cultured but is generally in the range of 22 to 28°C, preferably at about 25°C. The time period for the culture varies depending upon the anther cultured but is generally in the range of 0 to 14 days. Laser treatment should be done when microspore cells acquire the ability to devide and replicate DNA in callus induction medium, that is, the microspore cells morphologically change to cytoplasm-rich cells. Generally, the microspore changes to the cells proper for laser treatment within 2 weeks.

In addition, shed pollen cells obtained by the method of Ziauddin et al. [Plant Cell Reports, 9, 59 (1990)] may also be used.

Examples of Gramineas used herein include sweet corns, cereal plants, etc. Specific examples of the cereal plants are barleys, wheats, ryes, oats, etc. Barleys include Dissa, Igri, TRUMPF, CARINA, Haruna Nijo, etc. The anther may be collected from these plants in a conventional manner.

The microspore cells used are isolated from the anther.

The genetic substance bearing genetic information is the one controlled to stabilize its genetic information and express the genetic information in Gramineae. A specific example of the genetic substance is a plasmid which functions in Gramineae. Examples of the promoter which functions in Gramineae are promoters derived from cauliflower mosaic virus such as CaMV35S, CaMV19S, etc.; PR protein promoter, ADH-1 promoter, etc.; terminators such as CaMV19S, CaMV35S, NOS, etc. The genetic substance possesses, as the transformation properties, insect-resistant genes such as a desired BT toxin, protease inhibitor, etc.; foreign genes such as virus-resistant genes, genes for storage protein such as cazein, glutenin, etc, transposable genes such as Ac, Da, etc. In addition, the genetic substance may contain one or more genes encoding proteins which confer resistance to chemicals such as herbicide-resistant genes, antibiotic-resistant genes and function as an initial selection marker.

The solution containing the genetic substance may contain about 10 to 20,000 µg/ml of the gene desired to be transduced into the cell and other components, specifically, inactivated salts for promoting equilibration of an accurate osmosis or high tension, cell nutrients or other additives. A more specific example is a gene suspension containing 9 to 15% of mannitol.

Next, the laser device is focused on one of the pollen cells and the laser is excited to form pores in the cell envelop. Through the pores, the genetic substance is introduced into the cells. The size of a pore may be varied but should not be excessively large, as compared to the size of the cell. Specifically, the pore having a diameter of generally 5 to 500 nm is formed. The time period for applying the pulse is generally in the range of 5 to 20 nanoseconds, preferably 10 to 15 nanoseconds. The pulse energy is controlled generally in the range of 0.1 to 10 µJ. As the laser device, any optional device by which a laser can be focused on the appropriately fine focus may be generally used. Preferably, there may be used Hitachi Laser Cell Processor manufactured by Hitachi Ltd. which is commercially available as a device already utilized for laser microsurgery of mammal cells.

After the laser processing, the microspore cells are incubated in a solution containing the genetic substance for a time period sufficient for the genetic substance to disperse and permeate from said solution into the cells with pores. The time period for the incubation is generally 5 seconds to 2 hours and the temperature for the incubation is generally from 0 to 28°C.

After the laser processing and the incubation are carried out as described above, the resulting microspore cells or cells derived therefrom are cultured to form plants. A preferred embodiment of the present invention includes the method of Olsen et al. [Carlsberg Res. Commun., 52, 393 (1987)], the method of Ziauddin et al. [Plant Cell Reports, 9, 59 (1990)]. A further preferred embodiment includes the use of nurse cells.

The Examples illustrate the invention.

Example 1

After barley cv. Dissa was seeded, the barley was grown at 12°C for 16 hours in the light and at 10°C for 8 hours in the dark to collect the anthers of mid uninucleate stage microspores. The anthers were inoculated on modified MS + Ficol medium. After incubation at 25°C for 2 weeks, the anthers were opened with tweezers and a spatula to scrape the microspore cells out into DNA solution. After 10 to 20 anthers were taken in 1 ml of the solution, callus and deblis were removed through a nylon mesh of 96 µmø. By centrifugation (1000 rpm x 5 minutes), the solution was concentrated to 20 to 100 µl and recovered. The resulting concentrate was made a sample for processing with a laser pulse. The DNA solution used was composed of Okada solution + 15% mannitol + 10 µg/ml of pBI221 [marketed by Toyo Spinning Co.,

Ltd.], 10 µg/ml of pSBG102 (Hm^r) [β-glucuronidase structural gene of the aforesaid pBI221 is substituted with hygromycin B phosphotransferase structural gene [Gene, 25, 179 (1983)] between the site of BamHI and SstI] + 50 µg/ml of Calf Thymus DNA.

A drop of the DNA solution was placed on a Petri dish. In order to avoid drying, a 1% agarose piece of 5 mm square was put thereon. The Petri dish was covered and wound with a film, which was set in Hitachi Laser Cell Processor.

The sample includes microspore cells, developing single cells and cell mass which began to divide. The developing single cells which was cytoplasm-rich was chosen and pierced by a laser pulse with an energy of 0.5 V.

After the processing, the sample was diluted in 100 to 200 µl of aMS liquid medium and subjected to stationary culture at 25° C. Two weeks after, an equal volume of the medium (containing hygromycin B) was added. With respect to callus grown to have a diameter of several millimeters, transduction of GUS enzyme was examined. As a result, GUS activity was noted in 1/16 of selected calli.

Hereafter the method for assaying GUS activity is shown.

GUS Assay:

Composition of staining solution for gus assay

X-gluc solution (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) storage solvent [20 mg/l X-gluc DMF]

5 mg X-gluc in deoxynized DMF (dimethyl-formamide)

↓

solute in 5 ml of 50 mM K.P. buffer (potassium phosphate buffer) [pH 7.0, final concentration of 1 mg/ml]

GUS lysis buffer

50 mM K.P. buffer pH 7.0 + 10 mM EDTA + 0.1% Triton X 100 + 0.1% Sarkosyl + 10 mM 2-Mercaptoethanol

Preparation of cell:

When a small colony or the surface of tissue is stained, it may be impregnated with the solution.

The colony having a diameter of 100 µm was stained but it is questionable if substrate was incorporated in the plant.

Where the reaction is carried out quantitatively or accurately, a small amount of gus lysis buffer is added to the tissue, the mixture is mushed and substrate is added thereto. In the case of 1 mm callus, it is sufficient to use 20 µl of gus lysis buffer and 100 µl of X-gluc solution.

Example 2

After barley cv. Igri was seeded, the barley was grown at 12° C for 16 hours in the light and at 10° C for 8 hours in the dark to get the anthers included microspores of mid-uninucleate stage. The anther were inoculated on modified MS + Ficol medium. After incubation at 25° C for 2 weeks, the anthers were opened with tweezers and a spatula to scrape the pollen cells out into DNA solution. After 10 to 20 anthers were taken in 1 ml of the solution, callus and contaminants were removed through a nylon mesh of 96 µmø. By centrifugation (1000 rpm x 5 minutes), the solution was concentrated to 20 to 100 µl and recovered. The

resulting concentrate was made a sample for processing with a laser pulse. The DNA solution used was composed of Okada solution + 15% mannitol + 10 µg/ml of pBI221 [marketed by Toyo Spinning Co., Ltd.], 10 µg/ml of pSBG102 (hm^r) [β-glucuronidase structural gene of the aforesaid pBI221 is substituted with hygromycin B phosphotransferase structural gene [Gene, 25, 179 (1983)] between the site of BamHI and SstI] + 50 µg/ml of Calf Thymus DNA.

A drop of the DNA solution was placed on a Petri dish. In order to avoid drying, a 1% agarose piece of 5 mm square was put thereon. The Petri dish was covered and wound with a film, which was set in Hitachi Laser Cell Processor. The sample was treated automatically with an energy of 0.5 V.

After the processing, the sample was diluted in 500 µl of modified MS liquid medium and subjected to stationary culture at 25° C. Two weeks after, an equal volume of the hygromycin-selection medium (20 mg/l hygromycin B) was added. With respect to callus grown to have a diameter of several milimeters, transduction of GUS enzyme or hygromycin resistance was examined. As the result, GUS activity was noted in about 1/2000 of treated cytoplasm-rich cells.

Regeneration of plant:

Regeneration of transgenic barley

Leaf primordium corresponding to cotyledon was transplanted to rooting medium and cultured under the same conditions to promote development of the root and growth of shoot. A complete plant was regenerated in about a month. From the living leaves, nuclear DNA was isolated by the CTAB method (Plant Molecular Biology Reporter, 7:2, 116, 1989). Using 20 mers of the structural gene portion of marker gene as primers, it was attempted to conduct PCR (Science, 239:487, 1988), whereby DNAs equivalent to the respective genes were synthesized. From this, the presence of foreign genes in nuclear genome was confirmed.

By applying the present invention, the culture cells of Gramineae can be transformed. According to the present invention, it is unnecessary to prepare protoplasts and therefore, time and operations for transformation can be greatly reduced. Since haploid cells are transformed, the character transduced is conveyed without separating at a later generation. In addition, difficulties in experiments between species and cultivars are minimized so that it is easy to apply the present invention to practical species. According to the present invention, large pores can be formed as compared to the electroporation method so that DNA or substances having a large molecular weight can be introduced.

Claims

1. A method for transforming a monocotyledonous plant which comprises the steps of:
   (a) culturing an anther of said monocotyledonous plant in a callus induction medium; and
   (b) at a stage immediately before the microspore begins to divide or during the initial division, transducing a genetic substance into said microspore through a pore formed by a laser pulse.

2. The method according to claim 1 wherein said monocyteledonous plant belongs to the family of Gramineae.

3. The method according to claim 2 wherein said Gramineae is a cereal grass.

4. The method according to claim 3 wherein said cereal grass is barley.

5. The method according to any one of claims 1 to 4 wherein said pore formed by a laser pulse has a diameter of 5 to 500 nanometers.

6. The method according to any one of claims 1 to 5 wherein the time period for applying the laser pulse is in the range of 5 to 20 nanoseconds.

7. The method according to any one of claims 1 to 6 wherein the pulse energy applied is in the range of 0.1 to 10µJ.

8. The method according to any one of claims 1 to 7 wherein the time period for the transduction is in the range of 5 seconds to 2 hours.

9. The method according to any one of claims 1 to 8 wherein the temperature applied in step (b) is in the range of 0 to 28 °C.

10. The method of any one of claims 1 to 9 which comprises the additional step of:
(c) regenerating the transformed microspore into a complete plant.

11. The method of claim 10 wherein said plant is a non-chimeric plant.

12. The method according to any one of claims 1 to 11 which comprises the additional step of:
(d) expressing said genetic substance in said microspore and/or said plant.